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Original article

The expression of inducible nitric oxide synthase in human retinal pigment epithelial cells under stimulation of proinflammatory cytokine tumor necrosis factor-α

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ABSTRACT

Purpose: To elucidate the effects of tumor necrosis factor (TNF)-α on the expression of inducible nitric oxide synthase (iNOS) in retinal pigment epithelial cells in vitro.
Methods: ARPE-19 cell lines were cultured with TNF-α stimulation, and then treated with proteasome inhibitors (MG132 or lactacystin) for 30 minutes. The expression of iNOS was determined by RT-PCR and Western blot. The expression of nitric oxide (NO) was determined by an enzyme-linked immunosorbent assay. The interaction of nuclear factor kappa-B (NF-κB) activation and iNOS induction was assessed by electrophoretic mobility shift assay.
Results: The expression of iNOS in ARPE-19 was induced by TNF-α in a dose-dependent manner. Upregulation of iNOS resulted in increased production of NO. iNOS induced by TNF-α could be inhibited by MG-132 and lactacystin. Supershift assay revealed that NF-κB activation was responsible for iNOS induction.
Conclusion: TNF-α could induce iNOS expression and NO production in RPE cells, at least in part, via an NF-κB signal pathway.

1. Introduction

Nitric oxide (NO) is an inorganic, gaseous molecule which is synthesized in the retina by vascular, neuronal, and glial cells.[1,2] NO is synthesized by nitric oxide synthase (NOS). There are three forms of NOS: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS).[3,4] NO constitutively formed by eNOS and nNOS plays important roles in the regulation of physiologic functions, whereas NO produced by iNOS in excessive amounts leads to cytotoxicity, neurodegeneration, apoptotic cell death, and circulatory failure.[5–8] In the eyes, excess NO has been implicated in the pathogenesis of various disorders, including glaucoma, uveitis, and ischemic retinopathy[9–11] iNOS was shown to be induced *in vitro* in retinal pigment epithelial (RPE) cells and retinal Müller cells by various immuno-stimuli, such as interferon-γ (IFNγ), tumor necrosis factor-alpha (TNF)-α, and lipopolysaccharide (LPS).[12–14]

TNF-α participates in the pathogenesis of several inflammatory, neovascular, and degenerative ocular diseases.[15,16] Recent evidence has suggested that TNF-α may be an crucial player in the complex network of inflammation, linking angiogenesis and inflammation.[17] The biologic activities of TNF-α are performed through the TNF-α receptor. The TNF-α receptor was detected in the photoreceptors, RPE, and Müller cells; these structures can also express iNOS,[18–20] which implies that TNF-α may be involved in the regulation of iNOS expression in the retina. However, the exact mechanisms of TNF-α-induced iNOS expression in RPE cells has been unknown.

Nuclear factor kappa B (NF-κB) plays an important role in controlling inflammatory and immune responses.[21] In unstimulated cells, NF-κB is maintained in the cytosol as a heterodimer in complex with its inhibitory protein, IκB. When cells are stimulated, IκB is phosphorylated and degradated. This phosphorylation dissociates NF-κB from IκB and allows NF-κB to translocate to the nucleus, where it activates its target genes including iNOS.[22, 23] However, in RPE cells, the signaling mechanisms inducing iNOS by TNF-α association with NF-κB activation are not completely defined. Thus, in this study, we examined *in vitro* iNOS expression in ARPE-19 cells by the proinflammatory cytokine TNF-α, stimulation. Using NF-κB inhibitors, MG-132, and lactacystin, we sought to

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elucidate the role of NF-κB in the regulation of iNOS expression in RPE cells.

2. Methods

2.1. Cell culture

ARPE-19 cells were obtained from Bioresource Collection and Research (Hsinchu, Taiwan). The ARPE-19 cell line is an established cell line that has been shown to express many of the characteristics of RPE cells in culture. ARPE-19 cells were incubated in Dulbecco's modified Eagle's medium (DMEM, Biochrome KG, Berlin, Germany) supplemented with 10% (v/v) fetal bovine serum, 500 U/mL penicillin, and 500 μg/mL streptomycin. All cells were maintained at 37°C in a humidified 5% CO_2, 95% air incubator.

2.2. TNF-α, MG-132 and lactacystin treatment

ARPE-19 cells were seeded at a density of 10^5 cells/well in six-well plates, and grown to confluence. To investigate the dose response of TNF-α on iNOS mRNA expression, ARPE-19 cells were exposed for 4 hours to 10, 50, 100, and 200 ng/mL TNF-α, and medium alone (control). For NF-κB experiments, ARPE-19 cells were pretreated with 200 μM MG-132 (Calbiochem, Darmstadt, Germany) or 50 μM lactacystin (Cayman, Ann Arbor, MI, USA) for 1 hour and then incubated in new medium containing TNF-α 200 ng/mL for 4 hours.

2.3. Reverse transcription-polymerase chain reaction

Total RNA was extracted from ARPE-19 cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA (1 μg) was reverse transcribed to cDNA using 80 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Invitrogen) for 45 minutes at 37°C. Polymerase chain reaction (PCR) was performed in a reaction mixture of 50 μL containing 5 μL cDNA, 1 μL of sense and antisense primer, 200 μM of dNTPs, 5 μL 10 × Tag polymerase buffer, and 1.25 U Tag polymerase (Promega, Madison, WI, USA). Primer sequence for iNOS was forward primer 5′-ACAGGAGGGGTTAAAGCTGC-3′; reverse primer 5′-GCAGCTTTAACCCCTCCTGT-3′. Cycling parameters for iNOS amplification were as follows: 94°C for 60 seconds, 55°C for 60 seconds, and 72°C for 120 seconds. At the end of amplification, the reaction mixture was heated for 10 minutes at 72°C and then cooled to 4°C. A 10-μL sample of each PCR product was electrophoresed on 2% agarose gel containing ethidium bromide (Sigma, St Louis, MO, USA) in TAE buffer. The electrophoretic bands were documented and analyzed using an image analyzer (Digital 1D Science; Eastman Kodak, Rochester, NY, USA). GAPDH was used as a house keeping gene control.

2.4. Western blot analysis

ARPE-19 cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in 1 mL RIPA lysis buffer (Upstates, Lake Placid, NY, USA). Cell lysates were mixed 1:1 with Laemmli's sample buffer (Bio-Rad Laboratory, CA, USA) and boiled for 5 minutes. An equal amount of proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Piscataway, NJ, USA). Separated proteins were electrophoretically transferred from the gel to a PVDF membrane (Amersham and Pharmacia Biotech). The membrane was blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 for 1 hour at room temperature, and probed with anti-human fractalkine antibody (R&D Systems, Minneapolis, MN, USA) at 4°C overnight. After washing, the blots were incubated with secondary antibody coupled with horseradish peroxidase (Amersham and Pharmacia Biotech) for 1 hour at room temperature. Antibody-antigen complexes were then detected using enhanced chemiluminescence detection system (ECL, Amersham and Pharmacia Biotech) according to the manufacturer's instructions.

2.5. Measuring Nitric oxide production by ARPE-19 cells

Nitrite concentration was determined in the supernatant of cells and used as an index of NO synthesis. Nitrite was quantified colorimetrically after its reaction with Griess reagent using sodium nitrite as a standard. For measuring the nitrite concentration in cell medium, an equal volume of Griess reagent was added to the cell medium (0.5 mL), and the absorbance of the mixture was measured at 580 nm using a Beckman DU 640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA).

2.6. Electrophoretic mobility shift assay

ARPE-19 cells were harvested and washed, and nuclear protein were extracted with NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Co., IL, USA) according to the manufacturer's instructions. Equal amounts (10 μg) of nuclear protein extracts were subjected to a test for NF-κB protein/DNA binding using LightShift chemiluminescent electrophoretic mobility shift assay (EMSA kit, Pierce, Rockford, IL, USA) with a biotin end-labeled NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGG-3′) (Promega). Briefly, nuclear extracts were incubated in binding buffer with biotin end-labeled NF-κB oligonucleotide for 15 minutes at 4°C to allow DNA/protein binding. Specificity was determined by the addition of a 100-fold excess of unlabeled double-stranded NF-κB. To confirm the identity of NF-κB binding, supershift experiments were performed using anti-NF-kB p65 (Rel A; Rabbit) antibody (Rockland, Gilbertsville, PA, USA). The DNA/protein complexes were then resolved by a 6% native polyacrylamide gel electrophoresis in 0.5 × Tris-borate-EDTA buffer and transferred to a Hybond-N+ membrane (Amershan and Pharmacia Biotech). The biotin end-labeled DNA was detected using streptavidin-horseradish peroxidase conjugate, and a chemiluminescent substrate.

3. Results

3.1. TNF-α induced iNOS mRNA expression in ARPE-19 cells

To determine whether iNOS mRNA expression could be stimulated by TNF-α, we investigated iNOS mRNA expression in ARPE-19 cells stimulated with TNF-α for 4 hours. Treatment of ARPE-19 cells with TNF-α increased the expression of iNOS mRNA in a dose-dependent manner from concentration of TNF-α 0 to 100 ng/mL (Fig. 1). The effect of TNF-α-induced iNOS mRNA expression was decreased at a concentration of 200 ng/mL, when compared with a concentration of 100 ng/mL.

3.2. TNF-α induced iNOS protein expression in ARPE-19 cells

To further determine whether increased iNOS mRNA expression induced by TNF-α resulted in enhanced protein expression, ARPE-19 cells were treated with TNF-α for 4 hours, and iNOS protein expression was determined by Western blot analysis. The trends of iNOS protein expression were similar to that of iNOS mRNA expression. Exposure of ARPE-19 cells to TNF-α resulted in increased iNOS protein expression in a dose-dependent manner (Fig. 2).

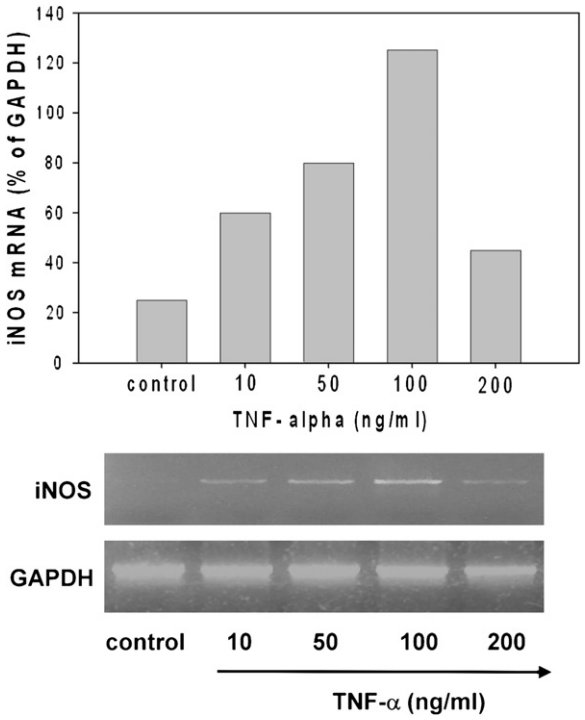

Fig. 1. TNF-α–induced iNOS mRNA expression in ARPE-19 cells. The upregulation of iNOS mRNA expressions in ARPE-19 induced by TNF-α was measured by reverse transcriptase-polymerase chain reaction (RT-PCR). ARPE-19 cells were stimulated with various concentrations of TNF-α (10, 50, 100, and 200 ng/mL) for 4 hours, and the expression of iNOS mRNA was quantified as a percentage of GAPDH.

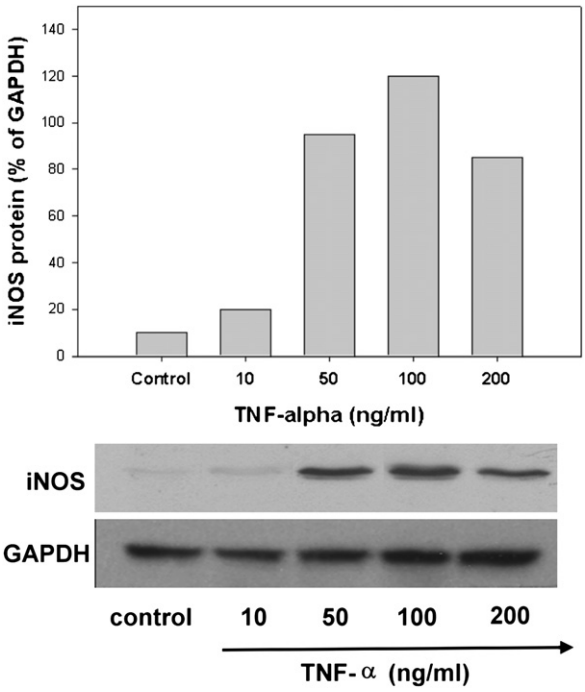

Fig. 2. TNF-α induced iNOS protein expression in ARPE-19 cells. The expression of iNOS protein in ARPE-19 induced by TNF-α was examined by Western blot analysis. ARPE-19 cells were stimulated with various concentrations of TNF-α (10, 50, 100, and 200 ng/mL) for 4 hours, and the expression of iNOS protein was quantified as a percentage of GAPDH.

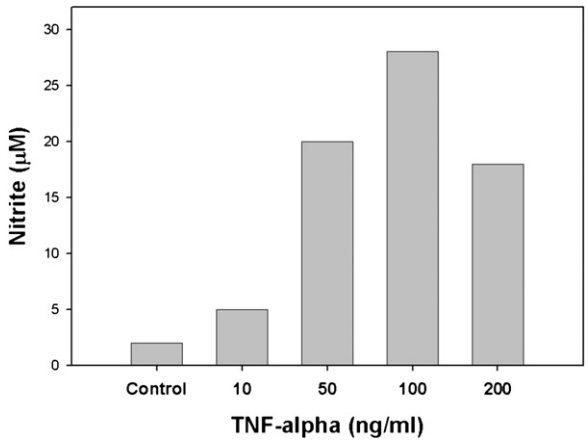

Fig. 3. TNF-α–induced nitric oxide expression in ARPE-19 cells. We analyzed NO expression in ARPE-19 cells by enzyme-linked immunosorbent assay (ELISA) kit. ARPE-19 cells were stimulated with various concentrations of TNF-α (10, 50, 100, and 100 ng/mL) for 4 hours, and subsequently the expression of NO was examined.

3.3. TNF-α induced nitric oxide expression in ARPE-19 cells

We investigated whether exposure of ARPE-19 cells to TNF-α could increase NO expression. Treatment for 4 hours with TNF-α also resulted in increased NO concentrations in a dose-dependent manner (Fig. 3).

3.4. MG-132 and lactacystin inhibited TNF-α induced iNOS mRNA and protein expression in ARPE-19 cells

We next investigated whether TNF-α–induced iNOS expression is NF-κB dependent in RPE cells. For this purpose, ARPE-19 cells were pre-incubated for 1 hour with MG-132 (200 μM), lactacystin (50 μM), or medium alone and subsequently cultured in new medium containing 100 ng/mL TNF-α for 4 hours. As shown in Fig. 4, TNF-α-induced iNOS mRNA expression in ARPE-19 cells was

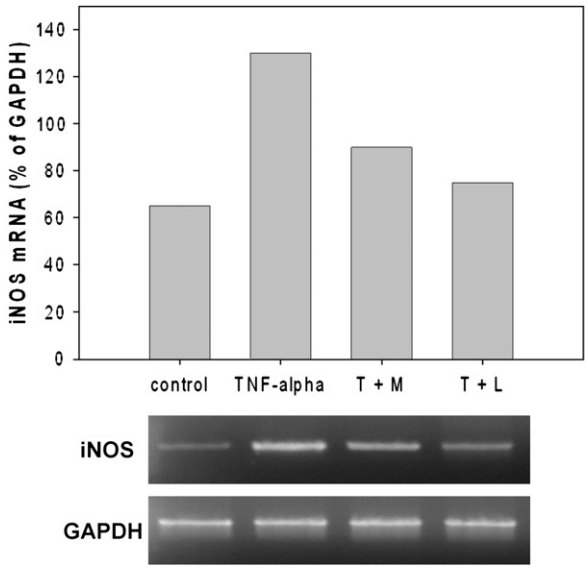

Fig. 4. MG-132 and lactacystin inhibited TNF-α induced iNOS mRNA expression in ARPE-19 cells. RT-PCR analysis of TNF-α induced iNOS mRNA expression in ARPE-19 cells in the presence of MG-132 or lactacystin. ARPE-19 cells were pretreated with MG-132 (200uM), lactacystin (50uM), or medium alone for 1 hour, and then stimulated with TNF-α (100 ng/mL) for 4 hours. Induction of iNOS mRNA by TNF-α was inhibited by MG-132 or lactacystin. T+M represents TNF-α + MG-132; T+L represents TNF-α + lactacystin.

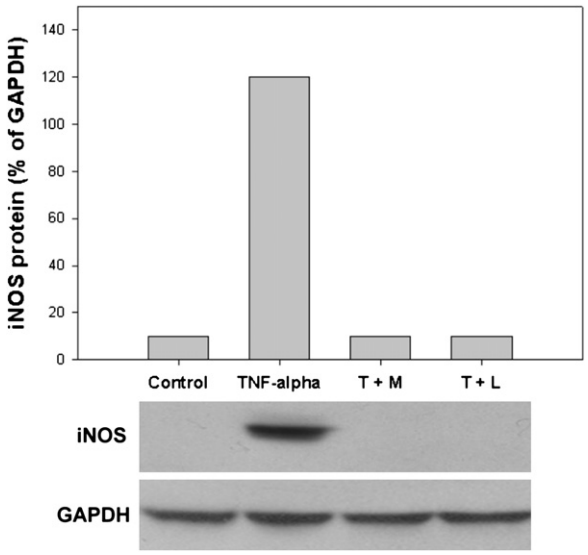

Fig. 5. Western blotting was performed to determine MG-132 and lactacystin effects on TNF-α induced expression of iNOS protein in ARPE-19 cells. ARPE-19 cells were pretreated with MG-132 (200uM), lactacystin (50uM), or medium alone for 1 hour, and then stimulated with TNF-α (100 ng/mL) for 4 hours. Induction of iNOS protein by TNF-α was inhibited by MG-132 or lactacystin. T+M represents TNF-α + MG-132; T+L represents TNF-α + lactacystin.

partially abolished by MG-132 or lactacystin treatment. Western blot analysis of iNOS also showed that TNF-α-induced iNOS protein expression in ARPE-19 cells was almost totally abolished by MG-132 or lactacystin (Fig. 5).

3.5. Detection of nuclear NF-κB complex in ARPE-19 cells stimulated with TNF-α by EMSA

TNF-α induced an increase in nuclear NF-κB complex. The addition of MG-132 and lactacystin inhibited NF-κB activities. The specificity of this reaction was confirmed by the addition of a 100-fold excess of unlabeled oligonucleotides, which eliminated the reactive band. The addition of antibodies directed against the 65-kDa subunit (p65) of NF-κB induced a supershift of the binding complexes (Fig. 6).

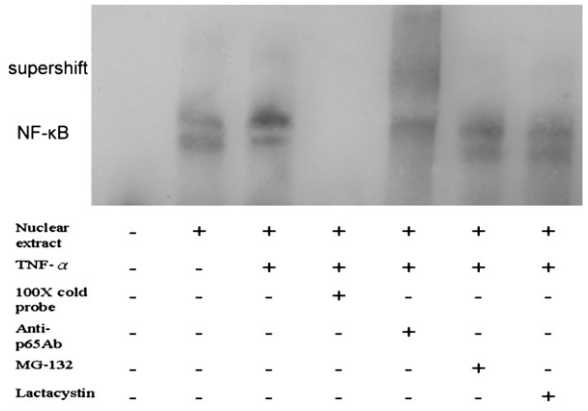

Fig. 6. Inhibitory effects of MG-132 and lactacystin on NF-κB activities induced by TNF-α in ARPE-19 cells was detected by electrophoretic mobility shift assay. The cultured ARPE-19 cells were pretreated for 1 hour with MG-132 (200 μM), lactacystin (50 μM), or medium alone, and subsequently maintained in TNF-α (100 ng/mL) for 4 hours. Nuclear extracts (10 μg) were obtained and incubated with biotin end-labeled NF-κB oligonucleotide. To test for specificity of NF-κB binding, we carried out supershift analysis with anti-p65 antibody, and competition experiments with a 100-fold excess of unlabeled oligonucleotides.

4. Discussion

In this study, we demonstrated that exposure of ARPE-19 to TNF-α resulted in a dose-dependent increase in mRNA and protein levels of iNOS. The upregulated iNOS may subsequently enhance the production of NO, and known potent angiogenic and inflammatory modulators that might contribute to inflammatory and angiogenic processes. Moreover, our results revealed that TNF-α-induced iNOS expression in ARPE-19 cells is mediated at least in part via activation of an NF-κB pathway.

TNF-α-induced iNOS expression has been demonstrated in a variety of cell types. Nakaizumi et al, showed that exposure to TNF-α increased the activation of NF-κB and the expression of iNOS in retinal ganglion cell (RGC)-5 line.[24] Shin et al, showed that TNF-α increases iNOS expression and leads to NO production in murine macrophages.[25] Our study extended the scope and showed TNF-α-induced iNOS expression in RPE cells. The expression of TNF-α is elevated in ischemia-induced retinal injuries, inflammation, diabetic retinopathy, and age-related macular degeneration (AMD).[26,27] TNF-α is a pivotal inflammatory mediator that activates leukocytes and promotes inflammatory cell migration. Moreover, TNF-α can induce cell apoptosis, differentiation, and activation. In this study, we found that TNF-α stimulated ARPE-19 cells to secrete NO. In RPE, NO contributes to the function of phagocytosis of rod outer segments and regulation of vascular endothelial growth factor gene expression. The secretion of NO by RPE cells may continue to promote and amplify the inflammatory and angiogentic responses.

Overexpression of iNOS appears to be a consequence of increased transcription. Several studies demonstrate that the promoter region of iNOS contain NF-κB binding sites.[28] Transcription factors NF-κB has been shown to control the transcription of iNOS genes in many cell types.[29] In this study, we found that TNF-α increased DNA binding activity of NF-κB in RPE cells. Furthermore, induction of iNOS mRNA expression by TNF-α was significantly inhibited by two NF-κB specific proteasomal inhibitors, MG-132, and lactacystin, which indicates that activation of NF-κB was involved in TNF-α-induced expression of iNOS. Several reports demonstrated that TNF-α can induce iNOS expression through activation of an NF-κB signal pathway.[30] Consistent with the findings of other groups, we found TNF-α-induced iNOS expression is, at least in part, via an NF-κB dependent pathway. The nuclear transcription factor NF-κB is a pivotal regulator of many important genes including multiple inflammatory chemokines. There are multiple factor binding sites in the iNOS gene promoter region and κB binding sites that bind with NF-κB are the most important.[31] From this point of view, NF-κB acts as a common regulator for various proinflammatory cytokines and may be a drug target for therapeutic applications, especially in ocular inflammatory and angiogenic diseases.

In conclusion, we report here that TNF-α-induced expression of iNOS and NO release in RPE cells were mediated through activation NF–κB. Understanding the signaling pathways of TNF-α in RPE cells may have significant therapeutic implications in developing a novel strategy against ocular inflammatory and angiogenic diseases.

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